Planta (2010) 232:1181–1189
DOI 10.1007/s00425-010-1251-5

ORIGINAL ARTICLE

Identifying differentially expressed genes in leaves of *Glycine tomentella* in the presence of the fungal pathogen *Phakopsora pachyrhizi*

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Received: 27 May 2010 / Accepted: 15 July 2010 / Published online: 14 August 2010

Abstract To compare transcription profiles in genotypes of *Glycine tomentella* that are differentially sensitive to soybean rust, caused by the fungal pathogen *Phakopsora pachyrhizi*, four cDNA libraries were constructed using the suppression subtractive hybridization method. Libraries were constructed from rust-infected and non-infected leaves of resistant (PI509501) and susceptible (PI441101) genotypes of *G. tomentella,* and subjected to subtractive hybridization. A total of 1,536 sequences were obtained from these cDNA libraries from which 195 contigs and 865 singletons were identified. Of these sequenced cDNA clones, functions of 646 clones (61%) were determined. In addition, 160 clones (15%) had significant homology to hypothetical proteins; while the remaining 254 clones (24%) did not reveal any hits. Of those 646 clones with known functions, different genes encoding protein products involved in metabolism, cell defense, energy, protein synthesis, transcription, and cellular transport were identified. These findings were subsequently confirmed by real time RT-PCR and dot blot hybridization.

Keywords *Glycine tomentella* · Suppression subtractive hybridization (SSH) · *Phakopsora pachyrhizi* · Defense responses · Resistance genes

Introduction

Soybean rust (SBR) is a serious disease causing crop losses in many parts of the world. SBR is caused by two fungal species, *Phakopsora pachyrhizi* and *Phakopsora meibomiae*. However, *P. pachyrhizi* is more aggressive than *P. meibomiae*. This former species has been identified in soybean production fields in the United States in 2004, and most US cultivars are reported to be highly susceptible to this fungal pathogen. Although fungicide use has been effective against SBR, developing SBR resistant cultivars would provide a sustainable and durable method of

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Electronic supplementary material The online version of this article (doi:10.1007/s00425-010-1251-5) contains supplementary material, which is available to authorized users.

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protection (USDA 2009). Thus, efforts have been underway to identify sources of genetic resistance to SBR in order to incorporate those into commercial cultivars of soybean.

Identifying sources of genetic resistance to SBR in *Glycine max* has not yielded promising results; whereas, sources of SBR genetic resistance have been identified in the wild perennial species *G. tomentella* (Hartman et al. 1992). To rapidly identify genes for resistance, the approach of suppression subtractive hybridization (SSH) has been used to isolate plant genes involved in resistance responses (Degenhardt et al. 2005; Xiong et al. 2001; Birch et al. 1999). A cDNA library generated by hybridization and subtraction will diminish the need for cloning of abundantly expressed housekeeping genes or genes commonly expressed in both infected and uninfected plants, and will contribute to normalizing cDNA expression profiles during library construction. Thus, this approach will allow for detection of genes of low-abundance and of differentially expressed transcripts (Degenhardt et al. 2005; Diatchenko et al. 1996).

In this study, mRNA expression profiles of a *G. tomentella* rust-resistant genotype (PI509501) and a rust-susceptible genotype (PI441101) have been analyzed to identify putative genes associated with host defense and/or resistance responses in *G. tomentella* in response to *P. pachyrhizi* infection using the SSH approach. Using this approach, several genes that are constitutively expressed at high levels in the resistant genotype, but transcribed only at low levels in the susceptible genotype have been identified. A number of these genes have been found to encode products belonging to groups of defense-related proteins.

Materials and methods

Plant material and *P. pachyrhizi* inoculation

Two *G. tomentella* genotypes, PI441101 and PI509501, were used as rust-susceptible and resistant genotypes, respectively. Both cultivars were grown as described previously (Soria-Guerra et al. 2010). Collection of SBR spores and inoculum preparation was done as previously described by Soria-Guerra et al. (2010). To infect detached leaves of 5-week-old plants, a spore suspension was diluted to 35,000 spores per 1 ml. For mock inoculation, 0.01% Tween-20 solution was sprayed using an air paint-brush (Paashe Airbrush Co., Lindenhurst, IL, USA) driven by a small compressor (Badger Co., Franklin Park, IL, USA) at 20 psi, at a rate of 1 ml per three leaflets. Following inoculation, leaflets were placed in the dark in a controlled environment growth chamber at 14/10 h of day/night (36 μmol m^{-2} s^{-1}) photoperiod at 22°C. All remaining leaflets that were not sampled were incubated under the same conditions and scored for rust symptoms at 12 days post-inoculation.

Sampling and RNA isolation

To study the expression of differentially expressed defense-related genes, SBR-infected and mock leaflets of rust-susceptible and -resistant genotypes were collected at 12 and 72 h following inoculation. All samples were immediately frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

Total RNA was extracted from frozen tissues using a modified cetyltrimethylammonium bromide (CTAB) method (Gasic et al. 2004), and stored at −80°C until needed. Briefly, 1 g tissue was ground into a fine powder in liquid nitrogen, and homogenized with 10 ml extraction buffer (2% CTAB, 2% PVP K-30, 10 mM Tris–HCl, pH 8.0, 25 mM EDTA, 2 M NaCl, and 0.5 g spermidine). After vortexing, tubes were incubated at 60°C for 15 min. Then, an equal volume of chloroform–isoamylalcohol (24:1) was added and centrifuged at 12,000*g* for 10 min at 4°C. The clear supernatant was transferred to a new tube, 7.5 M LiCl was added for precipitation at 4°C, and kept overnight.

SSH library construction

cDNA libraries were constructed using the PCR Select cDNA subtraction kit (Clontech, Mountain View, CA, USA). Briefly, cDNA was synthesized from total RNA with the Super SMART cDNA synthesis kit (Clontech, Mountain View, CA, USA) using a modified oligo dT provided by the manufacturer. cDNA was digested with restriction enzyme *Rsa*I to generate blunt-ended fragments. The tester cDNA pool was then subdivided into two halves, and each was ligated with a different cDNA adaptor provided in the kit (adaptor 1 and 2R). Each tester pool was then hybridized separately with an excess of driver cDNA, leading to the enrichment of differentially expressed sequences. During the second round of hybridization, the two primary hybridization samples were mixed together to form new double-stranded hybrids with different ends. Fresh denatured driver cDNA was added again to further enrich differentially expressed sequences.

Those fragments that present in the tester but not in the driver were then specifically amplified in two PCR amplifications. Primary PCR (30 cycles) and secondary PCR (15 cycles) amplifications were performed using primers that matched different adaptors to 5′ and 3′ ends, and according to the manufacturer's instructions. Purified PCR products from the second PCR reaction were cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* One Shot TOP10 cells (Invitrogen).

Colonies growing in LB medium containing 50 mg/L kanamycin were picked, and used to establish single-clone cultures in 96-well microtiter plates. Following overnight growth at 37°C, glycerol was added to a final concentration of 15%, and cultures were stored at −80°C. Plates were then forwarded to the W.M. Keck Sequencing Center (University of Illinois at Champaign-Urbana, IL, USA) to verify quality of cloned fragments by sequencing. A total of 1,536 clones were sequenced.

A total of four SSH libraries were constructed. The first library was constructed from the susceptible genotype PI441101 (GT-05), and a second library from the resistant genotype PI509501 (GT-06) at 72 h following inoculation, using mock inoculation tissue as the driver and rust-infected tissue as the tester. Another two SSH libraries were constructed using the susceptible genotype as driver and the resistant genotype as the tester at 12 (GT-02) and 72 h (GT-04) following inoculation.

Sequence analysis

Sequences were vector trimmed and assembled into contigs using Sequencher version 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA). For contig assembly, the rigorous data algorithm option was chosen with settings of a minimum match of 94% in a 50 bp overlap. A list of contigs and singletons was generated for each individual library. Each contig or singleton was assumed to represent a unique transcript. Unique transcripts were annotated by BLASTn from NCBI. Only those matches with E values below 10^{-5} were deemed significant. Functional categories of identified genes were assigned based on the data base of the Munich Information Center from Protein Sequence (MIPS) (http://mips.gsf.de).

Confirmation of gene expression by RT- PCR

A quantitative determination of the level of differentially expressed transcripts was carried out by real time RT-PCR. RNA was extracted as described above, and cDNA was synthesized using SuperScript III (Invitrogen) according to the manufacturer's instructions. Primer sequences for genes of interest are listed in Table 1. Primers for the tubulin transcript served as internal controls.

The SYBR Green real-time PCR assay was carried out in a total volume of 25 µl, containing 12.5 µl of 2× SYBR Green I Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µM of each of specific primers, and 100 ng template cDNA. The amplification program consisted of 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. Melting curves were obtained by slow heating at 0.5°C/s, from 60 to 90°C while continuously monitoring the fluorescence signal. A negative control without a cDNA template was run with each analysis to evaluate the overall specificity. Amplifications were carried out in 96-well plates in a 7300 Real Time PCR System (Applied Biosystems). All experimental samples were run in triplicate (technical replicates) with two biological replicates for each gene. Analysis of variance was used to determine statistical significance between samples. The quantification of gene expression was performed using the relative quantification (ΔΔCT) method and by comparing data with an internal control.

Preparation of digoxigenin-labeled DNA probes and dot blot hybridization

cDNA synthesized from mRNA isolated from rust-infected and mock leaflets of susceptible and resistant genotypes were used as templates for synthesizing DIG-labeled DNA probes. Probes were synthesized by random primed PCR using the DIG High Prime DNA Labeling and Detection Starter Kit (Boehringer Mannheim, Mannheim, Germany). Clones corresponding to defense genes of the four SSH were amplified by PCR using M13 primers. 2 µl of each PCR product was then dotted onto a positively charged nylon membrane. Following cross-linking for 5 min under ultraviolet light, pre-hybridization and hybridization were

Table 1 List of primers and predicted product sizes for RT-PCR confirmation analysis

Gene	Primer name	F (5′-3′)	R (5′-3′)	Size (bp)
AI966228	Apo5	ccagggaatcatagatgcggcga	cgaaccccaagttcttcagcctc	153
BE609114	GO-O	actacgcatctggtgcagaggac	tctgaaaggctgttggggcaa	174
BI974937	LLS	cctttcagaaaccaaggaaggtgg	gttgatggcaatggttggtcgctg	171
CD487711	Atpsyn	aaccattcccgctttcggtcagaa	tttccaaggtaaaagagcccccga	169
CO984181	Cathyd	tggcatcaacaacaccggattgg	aatccggtgttgttgatgcca	169
BU089724	GHMT	agctcctaccctcgagagtgg	tccccttggacctcgaagac	224
EH262452	Cytf	tggatatgcgtttgaaatgggtgc	cttggatagggaactatgcgagtg	186
FK003896	ARF	tgatggttggtcttgatgctgc	ggtgttctggaagtagtgcctcca	194
BE190908	Tfzinc	taccaacaggacaagccagca	cgcagaaacttgttgtgcattcc	219
Tubulin	Tub	gtgacttgaaccatctgatctcagc	gttgaagccatcctcaagccag	230

performed at 40°C. The probe was denatured at 100°C for 5 min prior to use. The following wash conditions were used: 2 × SSC + 0.1% SDS for 2 × 5 min at room temperature, followed by 0.1 × SSC + 0.1% SDS for 2 × 15 min at 68°C. Signal was detected using a CSPD substrate for alkaline phosphatase following the manufacturer's protocol.

Results

Identification of gene sequences

To identify those genes that are either similar or different between two genotypes, two SSH libraries, designated as GT-05 and GT-06 from susceptible (PI441101) and resistant (PI509501) genotypes, were successfully constructed using mock inoculation tissue as the driver and infected tissue as the tester. In order to identify genes expressed in the resistant but not, or at least expressed at lower levels, in the susceptible genotype, additional two SSH libraries, designated as GT-02 and GT-04, were successfully constructed using the susceptible genotype as the driver and the resistant genotype as the tester at 12 and 72 h following inoculation with *P. pachyrhizi,* respectively.

A total of 1,536 clones were sequenced. Assembly of the sequences using parameters of 94% match in a 50 bp overlap resulted in 195 separate contigs and 865 singletons. These sequences ranged from 1,300 to 85 bp in size, and were searched against the NCBI database, and fungal sequences were eliminated. Sequences of 160 clones (15%) were homologous to plant genes with unclassified functions, and most were hypothetical proteins. Moreover, 254 clones (24%) resulted in no hits, thus indicating that they had no homology to any sequences available in the current NCBI database (Table 2). These genes could be either deemed as unique to *G. tomentella* or that similar genes in other plant species have not yet been identified or deposited into the database. Otherwise, ~646 clones (61%) had high sequence similarity (*e* value) to genes of known functions (Supplementary Table 1).

Each identified sequence was then manually assigned to one of 14 functional categories or assigned as either unclassified or hypothetical categories. The functional categories were based on those available in the MIPS database. The number of genes and their frequency among the total number of genes in each of the four libraries is presented in Table 3. Among the known genes, those related to metabolism, cell rescue, and defense were overrepresented.

Transcripts related to cell cycle, cellular communication, cellular transport, sub-cellular localization, and protein fate were expressed at relative same level of abundance in all four SSH libraries (Table 3). For genes involved in energy, the GT-02 SSH library had the highest amount of transcripts, and it was almost similar to those in GT-04 and GT-05 SSH libraries. For those genes related to metabolism, they were similar in abundance in both GT-02 and GT-06 SSH libraries, but lower in abundance in the GT-05 SSH library. For genes involved in protein synthesis, high abundance of transcripts was observed in the GT-02 SSH library. Interestingly, the highest abundance of transcripts associated with cell rescue and defense as well as genes associated with binding functions were observed in the GT-06 SSH library, but this library also had the lowest numbers of genes related to transcription and energy (Table 3; Supplementary Table 1).

Taking into account the abundance of genes common among the three SSH libraries GT-02, GT-05, and GT-06, only 17 transcripts were found to be common among these libraries (Fig. 1). Among these transcripts, chitinases, heat shock proteins, oxidoreductases, ribosomal proteins, and several proteins related to binding functions were found. About 32, 38, and 27 transcripts were expressed in common between GT-02 and GT-06, GT-05 and GT-06, and GT-02 and GT-05, respectively. The remaining transcripts were specific to each of the SSH libraries (Fig. 1).

SSH on a susceptible *G. tomentella* genotype

The first subtractive cDNA library (GT-05) was constructed for the *P. pachyrhizi*, susceptible *G. tomentella* genotype (PI441101). A total of 384 independent clones were randomly chosen from this cDNA library that was enriched for genes expressed following inoculation with *P. pachyrhizi*. Sequences were grouped into 59 contigs and 262 singletons; whereas, 62 clones did not match any sequences present in the NCBI database. For the

Table 2 Clustering of transcripts from four cDNA libraries as described in "Materials and methods", *n* (%)

	Number of contigs	Number of singletons	Similar to known function genes	Similar to hypothetical protein	Similar to unknown function genes
GT-02	59	187	150 (60.9)	28 (11.4)	68 (27.7)
GT-04	33	186	108 (49.4)	50 (22.8)	61 (27.8)
GT-05	59	262	210 (65.4)	49 (15.3)	62 (19.3)
GT-06	44	230	178 (64.5)	33 (12.3)	63 (23.2)

Table 3 Abundance of transcripts in each functional category in each cDNA library, *n* (%)

Functional category	GT-02	GT-04	GT-05	GT-06
Cell cycle: Genes involved in mitotic cell cycle, cell cycle control, and DNA synthesis and replication	1 (0.4)	2 (0.9)	2 (0.6)	3 (1.2)
Cell fate: Genes related to cell growth/morphogenesis, dedifferentiation, dead cell and apoptosis	2 (0.8)	8 (3.6)	1 (0.3)	2 (0.4)
Cell rescue and defense: Genes associated with stress response, DNA repair, resistance proteins, defense related proteins, detoxification and cell death	13 (5)	17 (7.8)	35 (11)	40 (15)
Cellular communication: Genes associated with intracellular and intercellular communication, morphogenesis and receptor proteins	7 (2.8)	6 (2.7)	8 (2.5)	8 (3)
Cellular transport: Genes related to cellular transport activities (uni- and bidirectional, import and export)	8 (3.2)	9 (4.2)	15 (4.7)	8 (3.2)
Energy: Genes involved in glycolysis and gluconeogenesis, electron transport, respiration and photosynthesis	20 (8.2)	7 (3.2)	11 (3.4)	4 (1.5)
Metabolism: Genes associated with metabolism of compounds such as amino acids, nucleotides, carbohydrates, lipid, fatty acids, vitamins and secondary metabolism	40 (16.2)	17 (7.8)	51 (5.9)	48 (17.5)
Protein fate: Genes involved with protein folding, stabilization, protein targeting, translocation, modification proteolysis and storage	8 (3.3)	8 (3.6)	19 (5.9)	14 (5.2)
Protein synthesis: Genes related with ribosome biogenesis, translation, translational control, aminoacyl t-RNA synthetases	19 (7.7)	6 (2.8)	9 (3.2)	9 (3.2)
Protein with binding function: Genes whose main function is binding of specific ligands	18 (7.3)	9 (4.1)	17 (5.3)	30 (11)
Sub-cellular localization: Genes related to specific sub-cellular localization, regardless of their functional localization	1 (0.4)	5 (2.3)	13 (4.1)	3 (1.2)
Systemic interaction with the environment: Genes associated with plant/fungal specific systemic sensing and response, plant hormonal regulation, immune response	1 (0.4)	2 (0.6)	3 (1.2)	2 (0.4)
Transcription: Genes related to RNA synthesis, RNA processing and RNA modification	12 (5)	10 (4.6)	15 (4.7)	6 (2.2)
Various: Genes involved in cell type differentiation, development and regulation of metabolism	0	2 (0.8)	11 (3.4)	1 (0.2)
Hypothetical protein	28 (11.4)	50 (22.8)	49 (15.3)	32 (12)
Unknown	68 (27.6)	61 (27.9)	62 (19.2)	63 (23)

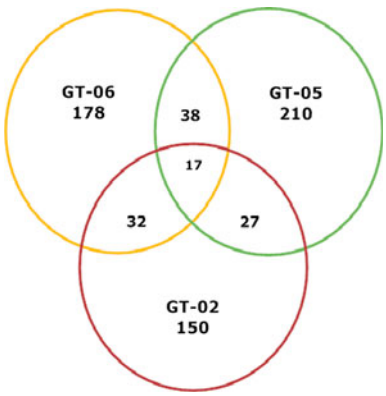

Fig. 1 Venn diagram illustrating co-expression patterns of sequences generated for three *Glycine tomentella* subtractive cDNA libraries. *GT-02* susceptible versus resistant genotype at 12 h, *GT-05* susceptible genotype, and *GT-06* resistant genotype

susceptible genotype, some genes that could be involved in defense against *P. pachyrhizi* were identified; i.e., genes coding for chitinases, glutation peroxidases, lipoxygenases, heat shock proteins, glutation S-transferase, and cytochrome P450.

Glutathione peroxidase and allene oxide synthase transcripts were also found only in this library, presumably expressed only in the susceptible genotype following infection with *P. pachyrhizi*. Moreover, larger numbers of lipoxygenases and heat shock proteins were found in GT-05 compared to the other three libraries. Among those transcripts expressed at high levels in GT-05 were cytochrome P450 monoxygenase, cysteine protease, dormancy-associated protein 1, expansin (EXLB3), and a single acid phosphatase transcript. Both Cystein protease and acid phosphatase have been reported to play regulatory roles, or at least are involved, in plant defense (Degenhardt et al. 2005).

SSH on a resistant *G. tomentella* genotype

A second subtracted cDNA library, GT-06, was constructed following inoculation of the resistant *G. tomentella* genotype (PI509501) with *P. pachyrhizi*. A total of 384 clones were randomly selected and characterized; while, 63 clones did not match any sequences present in the data base. For the *P. pachyrhizi*, challenged resistant genotype, higher numbers of differentially expressed genes, in comparison with the susceptible genotype, were found to be involved in cell rescue and defense. Among these, 4-coumarate-CoA ligase, monoxygenase, and pathogenesis-related gene 1 were

unique to this library. Likewise, alpha-dioxygenase, glutathione S-transferase, and oxidoreductase transcripts were detected at higher numbers in this library than in all other three libraries.

Pathogenesis-related genes are associated with the development of systemic acquired resistance (Fritig et al. 1998). It is now well established that the glutathione transferase is one of the major detoxification enzymes, with peroxidase and isomerase activities, thus playing an important roles in host resistance against pathogens (Sheenan et al. 2001). Abundance of transcripts of acidic chitinases, chitinases, and iron superoxide dismutase are detected at similar levels between GT-05 and GT-06 SSH libraries.

SSH on a *G. tomentella* genotype at 12 and 72 h after *P. pachyrhizi* infection

The third and fourth libraries, GT-02 and GT-04, were constructed using infected tissues as tester and uninfected tissues as driver at 12 and 72 h following *P. pachyrhizi* infection. A total of 384 clones from each library were selected and analyzed. At 12 h of infection (GT-02), an increase in the number of genes related to energy, metabolism, and protein synthesis was observed.

For genes related to cell rescue and defense, acidic chitinase, glycolate oxidase, and mellopeptidase were found only in GT-02; whereas, transcripts of glutathione S-transferase and iron superoxide dismutase were observed only in GT-04. Among other differentially expressed genes, those related to energy, including chlorophyll *a/b* binding protein, oxygen evolving enhancer protein 2 (OEE), light harvesting complex of photosystem I, and rubisco activase were found. Among these, OEE was highly expressed in GT-02, and a single transcript was also found in GT-06. The OEE has been related, through cross-talk, with defense functions (Yang et al. 2003). On the other hand, activation of rubisco activase has been reported to be induced following stress treatment (Hirotsu et al. 2005).

Overall, changes in differential expression of genes were detected more in GT-02, 12 h following *P. pachyrhizi* infection, than in GT-04, thus suggesting early responses to SBR in *G. tomentella*.

Validation of differential gene expression by RT-PCR and dot blot hybridization

To verify differential expression of selected transcripts, RT-PCR and dot blot hybridization were performed using total RNAs from susceptible-mock infected (SMI), susceptible-infected (SI), resistant-mock infected (RMI), and resistant-infected (RI) as templates. Genes selected for RT-PCR differential expression study included: apoptosis inhibitory 5 family protein (api 5 [AI966228]), glycolate oxidase (goo [BE609114]), lethal leaf spot 1-like protein (lls [BI974937]), ATP synthase (atsy [CD48771]), catalytic hydrolase (cah [CO984181]), glycine hydroxymethyltransferase (ghmt [BU089724]), cytochrome f (cf [EH262452]), small ARF-related GTPase (sARF [FK003896]), and transcription factor (tf [BE190908]). The soybean tubulin transcript was used as internal control. Expression levels of all these genes were higher in the tester than in the driver. Statistical significance of these differences was determined using analysis of variance (P values < 0.05) (Fig. 2).

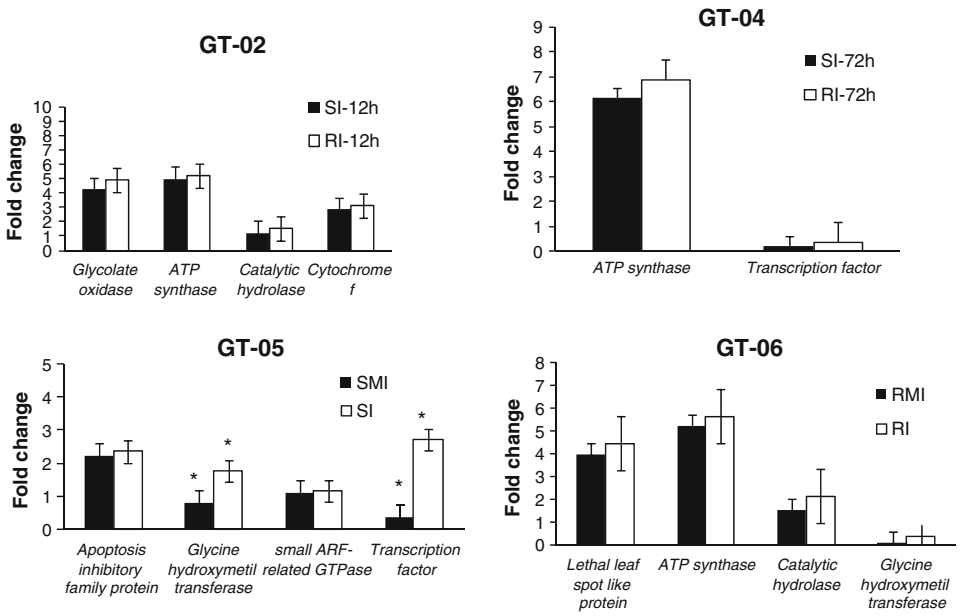

Fig. 2 Real-time RT-PCR confirmation analysis. *SI-12h* and *SI-72h* susceptible-infected genotype at 12 and 72 h post-inoculation, respectively, *RI-12h* and *RI-72h* resistant-infected genotype at 12 and 72 h post-inoculation, respectively, *SMI* susceptible-mock infected, *SI* susceptible-infected, *RMI* resistant-mock infected, *RI* resistant-infected. Statistically significant differences ($P < 0.05$) are denoted by *asterisks*

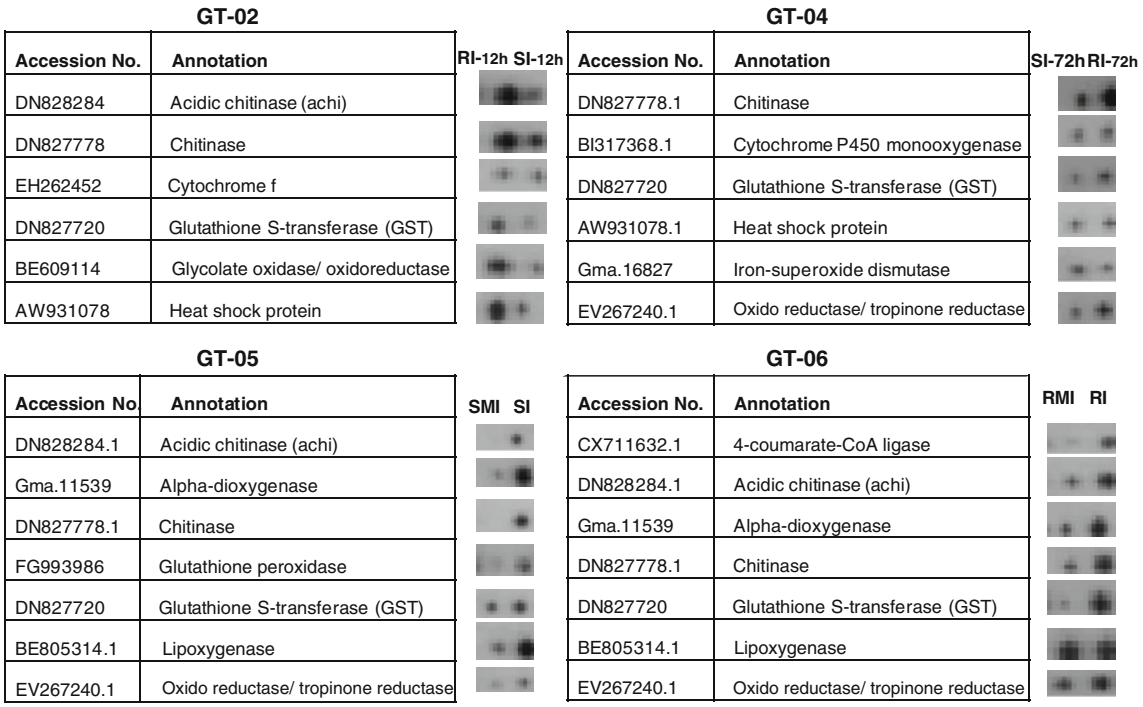

Fig. 3 Dot blot hybridization for a random set of defense genes. Blots spotted with PCR products from SSH were hybridized with probes prepared from selected cDNA clones from each library. *RI* resistant-infected, *SI* susceptible-infected, *SMI* susceptible-mock infected, and *RMI* resistant-mock infected

To validate the observed differential gene expression of SSHs, dot blot hybridization was performed using 52 PCR products that were chosen from the set of defense genes. Blots were hybridized with probes prepared from cDNA clones of each library. Nearly all of the 52 dots stained positively. In GT-02 and GT-04, over-expression of defense genes in RI over SI samples at 12 and 72 h, respectively, was observed (Fig. 3). In GT-05, expression was higher in SI over SMI samples; while in GT-06, expression in RI was higher than in RMI (Fig. 3). These findings validated the differential gene expression detected by SSH.

Discussion

A significant difference in the number and diversity of transcripts were observed in response to *P. pachirrizi* infection in the resistant (GT-06) versus susceptible (GT-05) genotypes, suggesting host responses to infection are genotype dependent.

The predominant defense-related transcripts found in all three libraries were chitinases. The consistently higher expression of the chitinase gene in both resistant and susceptible genotypes suggested that this gene might be involved in generalized defense responses. Chitinases have been reported to degrade fungal chitin following initial penetration of the pathogen into the intercellular space. Thus, the released chitin might then trigger a more generalized defense response in plant tissues (de A Gerhardt et al. 1997). Similarly, a heat shock protein transcript was found in all libraries. The induction of the heat shock protein gene expression has been well known and reported to be an important mechanism for plant acclimation under stress conditions via protein folding, assembly, translocation, and degradation (Mayer and Bukau 2005).

Energy-related transcripts were more highly represented in GT-02 that in the other constructed libraries. Some genes related to energy have been suggested to be involved in cross-talk with defense pathways (Sharma et al. 2009; Yang et al. 2003; He et al. 1998). In this study, these genes included chlorophyll *a*/*b*-binding protein, oxygen evolving enhancer protein 2 (OEE), light harvesting complex of photosystem I, and rubisco activase. The levels of transcripts involved in energy also supported the assumption that many of these transcripts were involved in *P. pachirrizi* resistance response. Yang et al. (2003) have demonstrated that OEE was phosphorylated by a protein complex containing a wall-associated kinase 1, a pathogenesis-related protein necessary for survival of plants during pathogen response.

In this study, metallothioneine transcripts were observed in GT-04 and GT-06. Previously, it has been reported that induction of metallothioneine-like proteins by insects,

wounding, and pathogen infection (Potenza et al. 2001; Choi et al. 1996). Moreover, it has been proposed that these metallothioneines could protect cellular constituents from oxidative damage (Choi et al. 1996). Therefore, a similar role for metallothioneines might be proposed for *G. tomentella* responses to *P. pachirrizi* infection.

A unique allene oxide synthase transcript is identified in the GT-05 SSH library. Allene oxide synthase has been reported to participate in the biosynthesis of jasmonic acid (JA), a widely occurring growth regulator that is also reported as a signaling molecule in plant defense (Song et al. 1993; Gundlach et al. 1992).

Biotic stress, such fungal pathogen attack, often results in lipid peroxidation of polyunsaturated fatty acids and involves an enzymatic mechanism driven by lipoxygenase (LOX) and a non-enzymatic mechanism driven by direct attack of reactive oxygen species (ROS). Accumulation of ROS may act as intermediate signaling molecules to regulate expression levels of genes involved in stress defense (Bajda et al. 2009; Mittler 2002). In this regard, elevated activity of several antioxidant enzymes, including oxide reductase (mainly in GT-06), glutathione peroxidase, and lipoxygenase (highly abundant in GT-05), superoxide dismutase, cytokinin oxidase, and glycolate oxidase, have been identified in libraries constructed in this study. Other genes related to oxidative stress, such as glutathione S-transferase (highly in GT-06) have been also isolated. This is consistent with previous studies using Affymetrix and oligo-based microarrays. Panthee et al. (2007, 2009) have analyzed transcriptome profiles of *P. pachyrhizi*-infected susceptible and resistant soybean plants and have reported on the up-regulation of lipoxygenase, gluthathione S-transferase, and peroxidase genes. Similarly, Choi et al. (2008) and Soria-Guerra et al. (2010) have also reported on the importance of peroxidases and lipoxygenases in soybean response to SBR infection.

Flavonoids are groups of secondary metabolites that are widely distributed in plants and play important roles especially in pigmentation and in defense responses, mainly through production of phytoalexins (Ververidis et al. 2007). In this study, a 4-coumarate-Co A ligase, an enzyme involved in the phenylpropanoid synthesis, is detected only in GT-06, and thus only expressed in the resistant genotype. Similar findings have been reported by Panthee et al. (2009) and Soria-Guerra et al. (2010) wherein this enzyme is expressed at higher levels in resistant rather than in susceptible *G. tomentella* genotypes in *P. pachyrhiz*-infected leaves. It has been reported that 4-coumarate-Co A ligase plays a role in the biosynthesis of jasmonic acid (JA), and there is str ong evidence supporting a central role of JA in plant defense (Schilmiller et al. 2007; Howe 2004).

In plant cells, oxylipins are compounds that play important roles in several physiological events including plant defense, development, and senescence (Mita et al. 2007). Thus far, identified phyto-oxylipins are synthesized via the lipoxygenase, cytochrome P450 monooxygenase, and α-dioxygenase pathways (Mita et al. 2007; Blee et al. 2002). Interestingly in this study, abundance of cytochrome P450 monoxygenase and lipoxygenase transcripts are higher in the susceptible genotype (GT-05) than in the resistant genotype (GT-06), whereas, the reverse is true for α-dioxygenase gene. In a previous study, cytochrome P450 monooxygenase and α-dioxygenase levels are higher in resistant that in susceptible G. tomentella genotypes (Soria-Guerra et al. 2010). According to Blee (2002), these enzymes are involved in preventing potentially harmful effects of free fatty acid accumulation during early response to pathogen attack in plants.

In this study, we report on the analysis of gene expression in the interaction between *G. tomentella* plants and the fungal pathogen *P. pachirrizi*, by comparing the responses susceptible and a resistant genotypes following infection. As a first step toward identification of genes differentially induced by infection, detached leaflets were used in these challenge studies, and represented a particularly suitable system. cDNA-subtracted libraries will be particularly valuable as they are enriched in genes involved in plant defense, and will serve as resources for future genetic enhancement efforts to introduce rust resistance into commercial soybean cultivars.

Acknowledgments This study was supported by a grant received from the United Soybean Board Project # 7262. Funds were also received from the Illinois Council for Food and Agricultural Research (C-FAR) project no. IDA CF07I-016-3-SEN. Partial funding was also received from CONACYT (Mexico) to support scholarship no. 81276 (C000/117/08).

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